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Reproductive Biology, Seed Formation and Vegetative Propagation of *Paeonia peregrina* and Ornamental Cultivars

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ABSTRACT: *Paeonia peregrina* Mill. is a medicinal plant of conservation concern in Bulgaria, where its petals and roots are harvested from wild populations for pharmaceutical use. Developing effective propagation methods is therefore essential for its conservation and potential cultivation. This preliminary study investigated selected aspects of the reproductive biology of *P. peregrina*, including pollination, seed formation, seedling development, and vegetative propagation. Field observations and experiments were conducted during 2024–2025 in a natural population near the town of Boboshevo, southwestern Bulgaria, while propagation trials were performed under *ex situ* conditions. To assess the role of pollinators, flowers were isolated from insect visitors during the bud stage. Pollinator exclusion resulted in almost complete reproductive failure, with only 0.74% seed set and the production of abortive seeds, whereas freely pollinated flowers achieved a mean seed set of 40.55%. Honeybees (*Apis mellifera*) were the most frequently observed pollen vectors, while bumblebees and ants were also recorded visiting flowers and buds. These findings indicate a strong dependence on insect-mediated pollination for successful seed production. Seeds sown immediately after ripening germinated in the second spring following sowing, demonstrating a prolonged dormancy period requiring sequential warm and cold stratification phases. Germination exceeded 50%, but seedling growth during the first year was slow. Irrigation and fertilization significantly improved the development of young plants, resulting in greater root biomass and larger underground organs after the second growing season. Vegetative propagation through division proved feasible and reliable, with 100% survival of daughter plants obtained from five-year-old specimens. However, the propagation coefficient was relatively low compared with the cultivated species *P. lactiflora*, reflecting the limited number of divisible vegetative buds. Nevertheless, transplanted and divided plants successfully regenerated under cultivation. The results demonstrate that *P. peregrina* relies heavily on pollinators for sexual reproduction and possesses slow but effective pathways for both seed and vegetative propagation. These findings contribute to the development of cultivation strategies that could support conservation efforts, reduce harvesting pressure on wild populations, and ensure a sustainable supply of medicinal plant material. Conserving both native habitats and pollinator communities is crucial to ensuring the long-term persistence of the species.

KEYWORDS: *Paeonia peregrina*; self-pollination; pollen vectors; vegetative and seed propagation specifics; Bulgaria

1 Introduction

Paeonia peregrina Mill. (Fig. 1) is a herbaceous perennial plant, usually found at altitudes from 0 to 1500 m in Albania, Bulgaria, Greece, Italy, North Macedonia, Moldova, Romania, Serbia and Turkey. The common or vernacular name of the species is червен (красив божур) [tzherven, krasiv bozhur] meaning red (beautiful) paeony in the Bulgarian language and црвен/и божур [crven/i božur] in the Serbian and North

Macedonian language also, referring to the red colour. In the Greek language, it is known as *Παιωνία η εκπατρισμένη* (Paionia i ekpatrismeni), while in Albanian it is *Bozhure*. It is also regionally referred to as *Lule bozhure* or *Lulegjaku*, which translates literally to “flower of blood”. The stems are unbranched. The leaves are doubly and triply divided. The flowers are single. The petals are obovate, sometimes toothed at the apex, and range in colour from dark or light red to pink or orange [1]. The petals and roots of *P. peregrina* are objects of commercial interest in Bulgaria for the pharmaceutical industry [2,3]. These plant substances contain mainly monoterpene glycosides, flavonoids, and phenolic acids, and are known for their analgesic, sedative, and anti-inflammatory properties [2]. The Ministry of Environment and Water (MOEW) of Bulgaria, annually controls the quantity of harvested petals and roots [kg] from the wild populations [3], due to the restricted resources of this species—up to 250 kg dry petals and up to 500 kg dry roots annually. The vulnerability of *P. peregrina* wild populations motivates the efforts for its introduction in cultivation. While the current global status of *P. peregrina* is Not Evaluated, the wild populations face severe threats—such as habitat loss and uncontrolled harvesting—in specific countries. Consequently, it is legally protected and designated as Endangered at the regional level in countries like Romania [4]. Propagation of *P. peregrina* is poorly studied [4,5]. The pollination of the species has not been studied so far.



Figure 1: *Paeonia peregrina* (a,b) in its native habitat in south-western Bulgaria.

Raising plants from seed *ex situ* represents an important step toward the future cultivation of this medicinal species, supporting its conservation and sustainable use. Additionally, vegetative propagation may provide an alternative approach for its cultivation and preservation.

The aim of this preliminary study is to investigate selected reproductive characteristics of *Paeonia peregrina*, including pollination biology, seed formation and vegetative propagation, as well as to comparatively assess the vegetative propagation of *P. peregrina* and cultivars *P. lactiflora* and *Paeonia* × *suffruticosa*.

2 Material and Methods

The experiments and observations were conducted during 2024–2025. The field experiments and observations were carried out near the town of Boboshevo (south-western Bulgaria, 42°09′22.26″ N

23°01'16.09'' E). The site comprises several stony, grassy more-or-less open habitats on carbonate rocks in or at the periphery of the mixed deciduous forest.

2.1 Spontaneous Self-Pollination Test

To assess the capacity for spontaneous self-pollination in *P. peregrina*, a total of nine flowers (one or two flower per plant) from seven plants (seven genets) were isolated from pollinators at the bud stage in a natural population in the vicinity of town of Boboshevo (Fig. 2). Flowers assigned to the pollinator-exclusion treatment were enclosed in custom-made fine-mesh pollinator-exclusion mini-cages. Each mini-cage consisted of a plastic cup with the bottom removed to form a rigid frame, which was covered with fine nylon mesh. The mini-cages were placed over individual flowers before anthesis and secured to the supporting stem, allowing air circulation and light penetration while preventing access by insect pollinators. A control treatment was included to verify that the pollinator-exclusion mini-cage itself did not affect fruit or seed development. For this treatment, two flowers were left open for unrestricted pollination, additionally hand cross-pollinated to ensure adequate pollen deposition, and subsequently enclosed in a fine-mesh pollinator-exclusion mini-cage. This control allowed us to determine whether any reduction in fruit or seed set observed in the pollinator-exclusion treatments resulted from the absence of pollinator visits rather than from any effects of the mini-cage on flower or fruit development. The formation of normal seeds indicated that the excluder does not negatively affect the process.

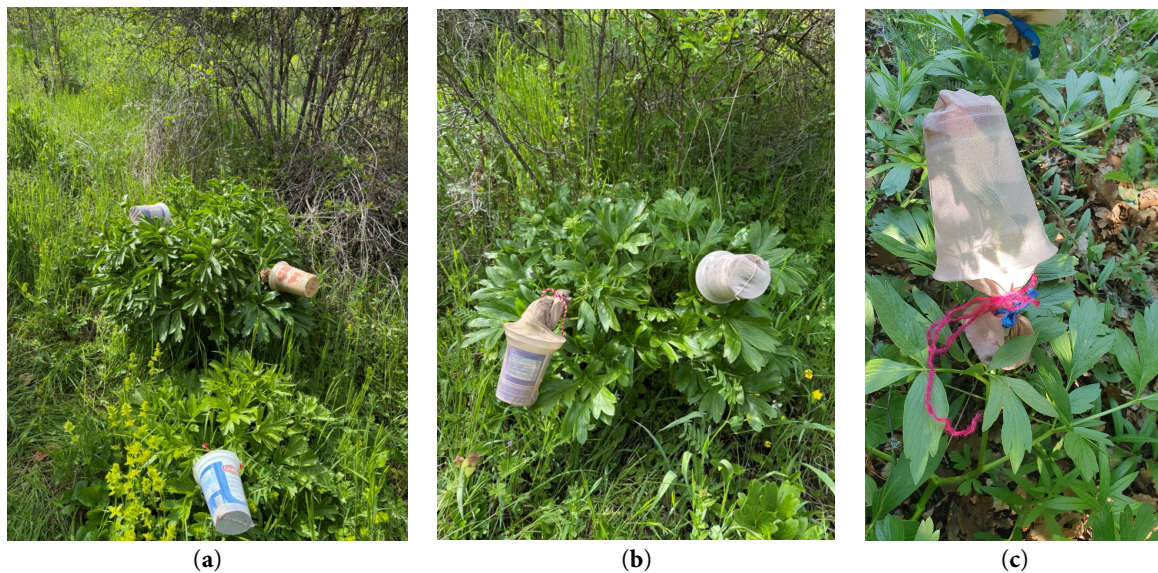


Figure 2: Buds (a,b) excluded from pollinators; (c) control.

2.2 Pollen Vectors

Pollinator composition and behaviour were studied in the native population in the vicinity of the town of Boboshevo during one observation session of 60 min each in four experimental plots (25 m² each).

2.3 Seed Propagation

At the beginning of August 2022, seeds of *P. peregrina* were collected from the collection of the Institute of Ornamental and Medicinal Plants (IOMP), Sofia, and were sown with the aim of obtaining a large number of plants for experiments focused on optimizing the cultivation technology of the species. The sowing was

conducted as a pilot experiment to assess feasibility. We used for the experiment 520 seeds. The seeds were sown in plastic trays on the surface of a substrate (peat + sand) and were covered with a 2 cm layer of washed sand. The containers with the seeds were kept outdoors in a shaded location under natural temperature conditions, and the substrate was maintained moist until germination occurred. The germination itself was not observed and counted since the seeds were covered by soil. At the end of the first growing season, the substrate was carefully sieved, allowing the underground seedlings to be recovered, counted, and measured before transplanting. Additionally, preliminary data were obtained from an experiment evaluating the response of young *P. peregrina* plants to different cultivation practices. A cultivation experiment was established using a split-plot design, with irrigation as the main-plot factor and fertilization as the subplot factor. Adjacent main plots were separated by 2 m, while subplots within each main plot were separated by 1 m. Four treatment combinations were evaluated: (i) no irrigation and no fertilization (control), (ii) no irrigation with fertilization, (iii) irrigation without fertilization, and (iv) irrigation with fertilization. The control treatment represented conditions most closely resembling the natural habitat of *P. peregrina* and served as the reference for evaluating the effects of irrigation and fertilization.

Each treatment initially comprised 60 young plants. The plants were transplanted in September, following completion of the first growing season, after the underground biomass of all individuals had been recovered, cleaned, measured, and recorded. The plants were then replanted according to the experimental design. Irrigation was supplied by a drip irrigation system and applied conservatively only when soil moisture deficit was observed. Fertilization consisted of the application of the complex fertilizer YaraMila Complex at a rate of 100 kg ha⁻¹ at the beginning of the growing season. Morphological traits were measured individually for each plant at the end of the growing season.

2.4 Vegetative Propagation

Several young plants were excavated from the natural population in the vicinity of the town of Boboshevo to examine their root system and assess their potential for vegetative propagation. Four plants of unknown age but assumed to be young were collected together with soil from their natural habitat and planted in a house garden in Sofia for observations on survival and ontogenesis *ex situ*. Two of them were planted in pots and another two directly in the garden.

Vegetative propagation was evaluated by dividing five 5-year-old *Paeonia peregrina* plants from the collection of the Institute of Ornamental and Medicinal Plants (IOMP). The results were compared with those obtained from five *P. lactiflora* plants of the same age. Plant division was performed in September, after the end of the growing season. The resulting daughter plants were immediately transplanted to field conditions and established in the IOMP collection in Sofia. Their survival and development were monitored over the following two growing seasons.

2.5 Comparative Assessment of the Underground Biomass

A comparative analysis of the underground parts of three species of the genus *Paeonia* was conducted to evaluate their productivity in root yield for pharmaceutical purposes. From plants harvested bare-root, intact roots were separated and measured; the total number of roots was recorded, and the total weight of the root system was determined. In *P. peregrina*, measurements were performed on five cultivated plants obtained through vegetative propagation, aged 5 years; in *P. lactiflora*, on 30 plants aged 5 years from different cultivars; and in *Paeonia × suffruticosa*, on 30 plants aged 5 years. The obtained data were subjected to analysis of variance (ANOVA) and *t*-test at a significance level of $p < 0.05$.

3 Results

3.1 Spontaneous Self-Pollination Test

The excluded flowers (N = 9) produced 32 follicles (Fig. 3a, Table 1). The experiment showed that the lack of pollinators did not prevent follicle formation. However, the resulting follicles were substantially smaller (Fig. 3a,b, Table 1). The test revealed that 99.3% of the ovules did not develop into seeds when pollinators were excluded (Fig. 3a,c, Table 1). Moreover, the seeds produced by isolated flowers appear to be abortive, exhibiting an unusually wrinkled seed coat, in contrast to the black, shiny, and smooth appearance of viable seeds. (Fig. 3c,d).

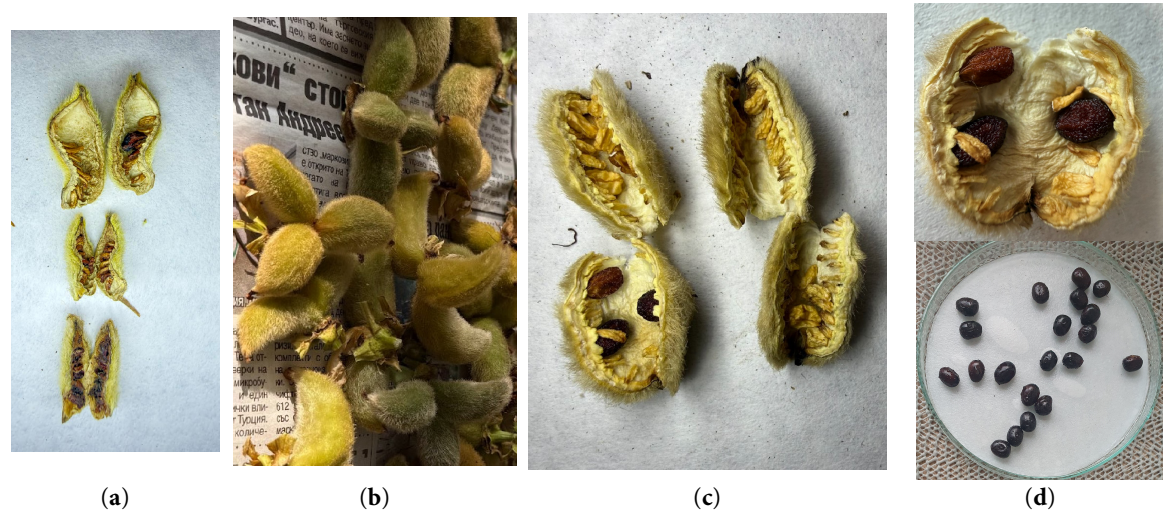


Figure 3: *Paeonia peregrina* follicles (a) in result of free pollination; (b,c) in result of pollinators’ exclusion, (d) abortive ovules and seeds formed without pollen vectors versus ripe seeds resulting from free pollination.

In comparison the hand-pollinated flowers, which were also covered with pollinator excluders, produced normal seeds (Table 1). This demonstrates that the absence of seed formation in the isolated flowers was caused by the lack of spontaneous self-pollination or self-incompatibility, rather than by the experimental treatment itself.

The freely pollinated flowers in the native population produced significantly more ripe seeds per follicle ($N = 4.29 \pm 3.15$) than isolated flowers, which produced no ripe seeds (Table 1). The free pollination also significantly reduced the number of undeveloped ovules (10.58 ± 2.56 vs. 18.52 ± 3.18). Average seed set increased from 0.74% in isolated flowers to 40.55% in freely pollinated flowers. Consequently, *Paeonia peregrina* depends on pollen vectors for its seed production.

Table 1: Seed set as a result of free pollination and self-pollination test in the wild population.

Formation of seeds as a result of free pollination				
	Ripe seeds	Abortive seeds	Undeveloped ovules	% of seed set
Average	4.29	0	10.58	40.55%
Standard deviation	3.15	0	2.56	
Min	0	0	5	
Max	9	3	19	

Table 1: *Cont.*

Formation of seeds from isolated flowers				
	Ripe seeds	Abortive seeds	Undeveloped ovules	% of seed set
Average	0	0.138	18.52	0.74%
Standard deviation	0	0.571	3.18	
Min	0	0	12	
Max	0	3	24	

3.2 Pollen Vectors

This preliminary study revealed that *Paeonia peregrina* has numerous stamens and honeybees were observed gathering pollen in its flowers (Fig. 4a). Honeybees were observed visited the flowers even before full anthesis (Fig. 4b). Although visits were relatively rare, one to four honeybee workers were simultaneously observed within a single flower. A worker of *Bombus* cf. *lapidarius* was also recorded as a pollen vector, although its visitation frequency was lower than that of honeybees.

Additionally, the buds of *P. peregrina* were frequently and abundantly visited by large numbers of ants due to excess of extrafloral nectar.

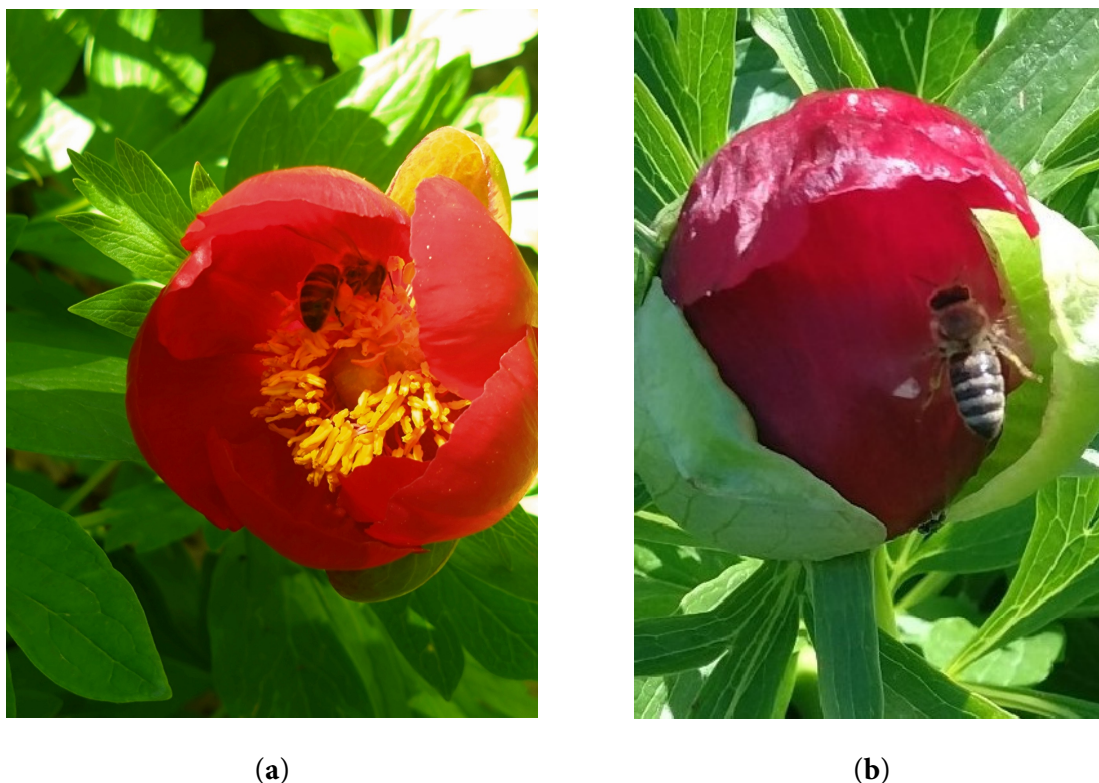


Figure 4: Honeybees serving as pollen vectors of *Paeonia peregrina* (a) pollen gathering (b) attracted by extrafloral nectar.

3.3 Seed Propagation

Seeds of *Paeonia peregrina* sown immediately after ripening germinated in the second spring following sowing. This indicates the presence of a prolonged dormancy period requiring sequential warm and cold stratification. Under natural conditions, seeds experience warm stratification until the onset of the first winter, followed by cold stratification during winter, a second warm stratification period during the

subsequent summer, and a further cold stratification phase during the second winter before germination occurs. The extended duration of this process presents practical limitations for cultivation and propagation, highlighting the need for methods that can accelerate dormancy release and germination. Although the germination percentage was not quantified in this preliminary research, it was estimated to exceed 50%.

The seedlings did not appear above the ground the first year after germination (Fig. 5). Young plants exhibited minimal growth during the first growing season, and by its end the underground part had a fresh weight of 491.94 ± 246.48 mg, a length of 48.25 ± 8.8 mm, and a diameter of 3.8 ± 1.2 mm (data obtained from a sample of 250 plants).

At the end of the second year after germination, the roots of the plants showed the following parameters presented in Table 2. The results indicate that young plants respond positively to both irrigation and fertilization, as evidenced by increased root biomass and improved growth parameters. These findings suggest that cultivated *P. peregrina* plants may achieve substantially higher productivity than individuals growing in natural habitats, supporting further efforts to develop efficient cultivation practices for the species. However, the extent to which cultivation conditions and genetic factors affect the quality and phytochemical composition of the harvested plant material remains unknown and warrants further investigation.



Figure 5: Roots at the end of the first vegetation season.

Table 2: Main biometric parameters of the underground parts of *P. peregrina* plants after the second growing season.

	Fresh Weight (mg)	Length (mm)	Diameter (mm)	Number of Buds
Non-irrigated conditions	681 ± 117^a	30.47 ± 3.0^a	4.47 ± 0.7^a	1.66 ± 0.5^b
Non-irrigated conditions + fertilization	871 ± 293^b	35.38 ± 4.1^b	4.95 ± 0.9^b	1.38 ± 0.5^a
Irrigated conditions	920 ± 261^b	35.7 ± 3.0^b	5.23 ± 0.8^b	1.71 ± 0.7^b
Irrigated conditions + fertilization	1311 ± 412^c	38.7 ± 3.3^c	5.81 ± 0.7^c	2.42 ± 0.7^c

Note: Values are presented as mean $\pm$ standard deviation. Within each column, different superscript letters (a, b, c) indicate statistically significant differences among treatments at $p < 0.05$ according to multiple pairwise *t*-tests. Values sharing the same letter are not significantly different.

3.4 Vegetative Propagation

The apparently young plants of unknown age collected from the wild population revealed an atypical rhizome, with some of the lateral roots fusiform (spindle-shaped, wide in the middle and narrowing at both ends) or tuberous. Examination of the underground structures of these plants indicated that each individual could potentially be divided into two to six daughter plants. Of the four plants transplanted from the wild, only the two individuals planted directly in a garden in Sofia survived. Although their aboveground parts gradually senesced after transplantation, new shoots emerged the following spring. The two individuals were planted in pots did not survive. None of the surviving plants flowered during the two growing seasons after transplantation. Monitoring is ongoing (Fig. 6).

As plants age, additional structures bearing vegetative buds develop, increasing the potential for division. The results from the Institute of Ornamental and Medicinal Plants (IOMP) experimental collection indicate that vegetative propagation is a reliable but relatively slow method for producing new *P. peregrina* plants. All daughter plants survived during the study period. However, both the propagation coefficient and the growth rate of daughter plants were lower than those of the widely cultivated *P. lactiflora* (Table 3). This low propagation coefficient is mainly due to the concentration of vegetative buds within a small stem-like structure that can be divided into only a limited number of segments, regardless of root system size. These observations suggest that annual division at the end of each growing season may be more effective than dividing older, larger plants after several years of growth. Some daughter plants flowered in the year following division. During intensive propagation, flower removal is recommended to prevent the diversion of resources to seed production and promote vegetative growth.

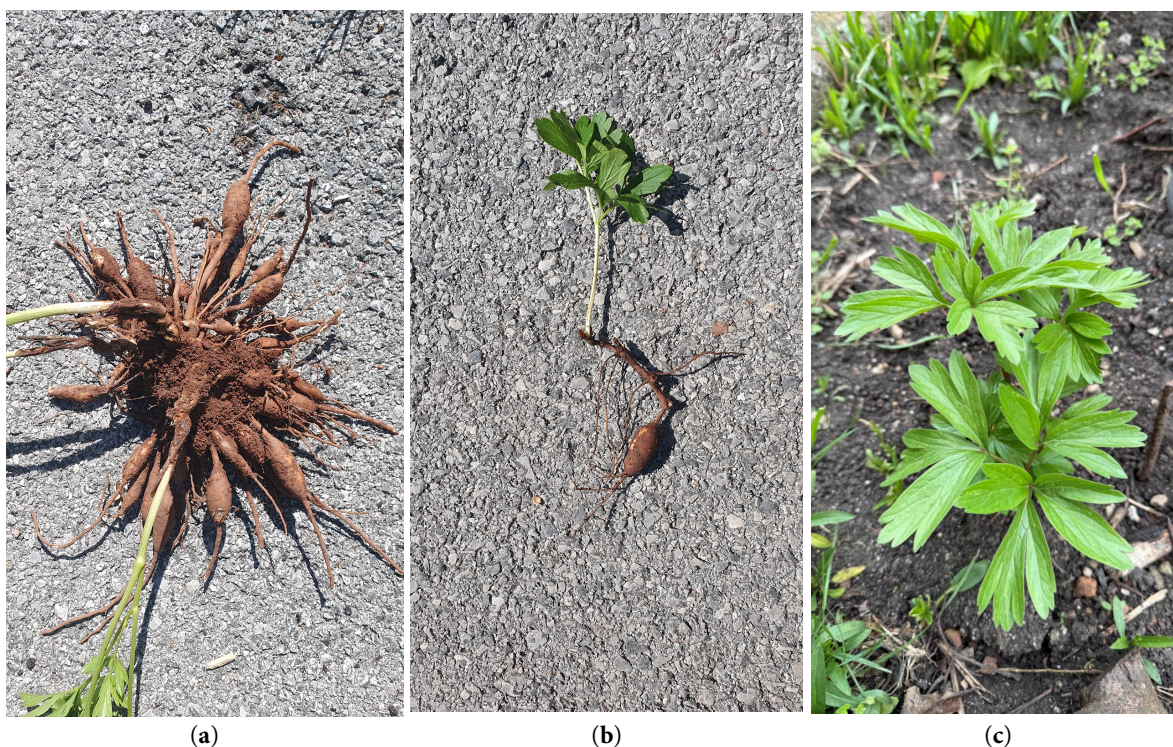


Figure 6: Potential for vegetative propagation of *P. peregrina*: (a) atypical rhizome, with some of the lateral roots; (b) objects for transplantation; (c) transplanted individual during the next vegetation season.

Table 3: Data on the division of 5-year-old plants of *P. peregrina* and *P. lactiflora* and the development of the resulting daughter plants. Different letters (a,b) within the same column indicate significant differences at the 5% level ($p < 0.05$), determined by multiple *t*-tests.

Species	Number of Plants	Average Number of Daughter Plants	Daughter Plants Surviving until the End of the First Growing Season	Average Number of Stems Per Daughter Plant	Average Number of Stems
				1-year	2-year
<i>P. peregrina</i>	5	2.4 ± 0.54^a	100%	2.6 ± 0.5^a	3.5 ± 1^a
<i>P. lactiflora</i>	5	7.8 ± 1.3^b	94.9%	4.9 ± 1.3^b	7.4 ± 1.3^b

3.5 Comparative Assessment of the Underground Biomass

The root systems of *Paeonia peregrina*, *P. lactiflora*, and *P. × suffruticosa* were compared to assess differences in root biomass and morphology (Table 4). The study was conducted using five-year-old plants from the collection of the Institute of Ornamental and Medicinal Plants (IOMP). Among the three species, *P. lactiflora* exhibited the highest total root system mass. However, *P. peregrina* plants are considerably smaller and can therefore be cultivated at a much higher planting density, which may partly compensate for their lower individual root yield. Furthermore, the ability of *P. peregrina* to be propagated from seed may facilitate the large-scale production of planting material. In contrast, propagation of *P. lactiflora* relies almost exclusively on vegetative division.

Table 4: Characteristics of the root system of 5-year-old plants of *P. peregrina*, *P. lactiflora*, and *P. × suffruticosa*.

Paeonia	Average Fresh Root Weight (g)	Average Root Length (mm)	Root Diameter (mm)	Average Number of Roots Per Plant	Average Root System Weight (g)
<i>P. peregrina</i>	3.02 ± 0.26	114.68 ± 32.29	5.23 ± 0.80	33.26 ± 2.45	100.5 ± 23.43
<i>P. lactiflora</i>	47.84 ± 11.70	216.48 ± 23.66	16.13 ± 2.67	16.94 ± 2.74	810.17 ± 198.13
<i>P. × suffruticosa</i>	8.26 ± 1.73	328.35 ± 25.21	8.97 ± 1.35	19.16 ± 2.37	158.26 ± 31.45

4 Discussion

Our field results indicate that *Paeonia peregrina* is pollinator-dependent, as flowers isolated from pollinator access failed to produce seeds. This observation is consistent with studies on other species of the genus *Paeonia*. For example, *P. officinalis* is partially self-incompatible and demonstrates only a limited ability to achieve autonomous self-pollination without pollinator visitation [6]. *P. officinalis* is a close relative to *P. peregrina*. Research reveals that *P. officinalis* is a homoploid hybrid species derived from hybridization between the allotetraploid species *P. peregrina* and a member of the *Paeonia arietina* species group [7]. Additionally, reproductive success in several other *Paeonia* species depends strongly on insect-mediated pollen transfer. Previous studies have identified bees as the primary pollinators and have shown that seed production is frequently limited by the efficiency of pollen transfer, indicating partial self-incompatibility and/or a limited capacity for autonomous self-pollination [8–11]. Some of the *Paeonia* species such as are completely self-incompatible [12,13]. Therefore, the global decline of pollinators [14,15] may jeopardize seed reproduction in *Paeonia* species in general and in *P. peregrina* in particular and ultimately threaten the long-term persistence of natural populations. Promote organic farming practices and reduce the use of synthetic agricultural inputs, as these measures can enhance pollinator diversity and abundance, thereby

strengthening pollination services and improving seed production in both agricultural and adjacent natural ecosystems [16].

The pollinator observations in the studied population were performed on a limited observation period. Nevertheless, they showed that pollen-collecting insects, mainly honeybees and occasionally bumblebees, visit the flowers of *P. peregrina*. Similar pollination systems have been reported for other *Paeonia* species, whose flowers provide abundant pollen but little or no nectar, thereby attracting insects that collect pollen as the main floral reward. *Apis mellifera* (honeybees) and bumblebees (e.g., *Bombus* spp.) are considered the most effective pollinators. Additionally, solitary bees such as *Andrena* spp. and *Lasioglossum* spp. frequently visit *Paeonia* species to collect pollen as well as Syrphid flies (hoverflies) [8–10,12,17–20].

Peony flowers have extrafloral nectaries to provide food for ants and in turn, the ants protect the buds from other floral-feeding insects [21]. The phenomenon is well known for *P. lactiflora* [22] but not reported for *P. peregrina* before.

Propagation of *P. peregrina* is poorly studied however, as in other species of the genus, seed propagation is likely constrained by complex dormancy mechanisms. These involve both an impermeable seed coat and an underdeveloped embryo [4,5]. The simple mechanical treatments such as scarification and seed coat removal are insufficient to induce germination and break the deep morphophysiological dormancy of *P. peregrina* seeds [23]. In addition to sexual reproduction, vegetative persistence may contribute to the stability of natural populations. The observed root morphology of *P. peregrina*, including fusiform and tuberous lateral roots, may support regeneration and long-term survival of individual plants. Although vegetative propagation does not contribute to genetic diversity within populations, it may facilitate population persistence under conditions where seed production is limited by pollinator availability or other ecological constraints [6,8,10,11,24].

Compared with previous studies on the reproductive biology of *Paeonia* species, the present work provides several novel contributions. First, this is the first field-based study to demonstrate the strong dependence of *Paeonia peregrina* on insect-mediated pollination under natural conditions in Bulgaria using pollinator-exclusion experiments. Second, the study identifies the principal flower visitors of *P. peregrina*, documenting *Apis mellifera* as the dominant pollen vector, with occasional visits by bumblebees and frequent attendance of ants to extrafloral nectaries. Third, unlike previous studies that have mainly focused on seed dormancy and germination, this work integrates observations on pollination biology, seed production, seedling establishment, vegetative propagation, and cultivation practices within a single study. Fourth, preliminary cultivation experiments demonstrate that irrigation and fertilization substantially improve early root development and biomass accumulation, providing practical information for future cultivation of this medicinal species. Finally, the comparative assessment of vegetative propagation efficiency and underground biomass between *P. peregrina* and cultivated peony species offers new information relevant to conservation, sustainable cultivation, and pharmaceutical production. Collectively, these findings expand current knowledge of the reproductive biology of *P. peregrina* and provide a scientific basis for developing conservation-oriented cultivation strategies.

5 Conclusion

The results demonstrate that *Paeonia peregrina* is strongly dependent on insect pollinators for successful seed production. Seed propagation is constrained by prolonged dormancy and slow seedling development, while vegetative propagation, although reliable, has a relatively low multiplication rate. These biological characteristics, together with the vulnerability of natural populations, highlight the importance

of conserving both native habitats and pollinator communities to ensure the long-term persistence of the species.

This preliminary study identifies several priorities for future research, including the optimization of seed germination and vegetative propagation techniques, as well as the development of efficient cultivation practices. Successful cultivation would reduce harvesting pressure on wild populations and contribute to a stable supply of high-quality plant material for pharmaceutical use.

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Availability of Data and Materials: The authors confirm that the data supporting the findings of this study are available within the article.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

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